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Review

Pathogenicity, population genetics and dissemination of *Bacillus anthracis*

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ABSTRACT

Bacillus anthracis, the etiological agent of anthrax, procures its particular virulence by a capsule and two AB type toxins: the lethal factor LF and the edema factor EF. These toxins primarily disable immune cells. Both toxins are translocated to the host cell by the adhesin-internalin subunit called protective antigen PA. PA enables LF to reach intra-luminal vesicles, where it remains active for long periods. Subsequently, LF translocates to non-infected cells, leading to inefficient late therapy of anthrax. *B. anthracis* undergoes slow evolution because it alternates between vegetative and long spore phases. Full genome sequence analysis of a large number of worldwide strains resulted in a robust evolutionary reconstruction of this bacterium, showing that *B. anthracis* is split in three main clades: A, B and C. Clade A efficiently disseminated worldwide underpinned by human activities including heavy intercontinental trade of goat and sheep hair. Subclade A.Br.WNA, which is widespread in the Northern American continent, is estimated to have split from clade A reaching the Northern American continent in the late Pleistocene epoch via the former Bering Land Bridge and further spread from Northwest southwards. An alternative hypothesis is that subclade A.Br.WNA. evolved from clade A.Br.TEA tracing it back to strains from Northern France that were assumingly dispatched by European explorers that settled along the St. Lawrence River. Clade B established mostly in Europe along the alpine axis where it evolved in association with local cattle breeds and hence displays specific geographic subclusters. Sequencing technologies are also used for forensic applications to trace unintended or criminal acts of release of *B. anthracis*. Under natural conditions, *B. anthracis* generally affects domesticated and wild ruminants in arid ecosystems. The more recently discovered *B. cereus* biovar *anthracis* spreads in tropical forests, where it threatens particularly endangered primate populations.

1. Introduction

Bacillus anthracis is a spore forming Gram-positive bacterium causing anthrax. It has a long history as a life threatening infectious agent of humans and animals worldwide (Turnbull, 2002). However, active control measures, particularly strict prohibition of burial of carcasses and slaughter waste as well as large animal vaccination programs during the first part of the 20th century, significantly reduced the incidence of anthrax in many industrialized areas. Anthrax is potentially infectious to most mammals. However, it primarily affects ruminants since they are most frequently exposed to the pathogen in the environment. This bacterium survives for decades as spores on meadows contaminated by abandoned or buried carcasses of antecedently succumbed animals. Humans generally acquire anthrax by contact with infected animals or from occupational or nutritional exposure to contaminated animal products such as meat, hair or skin (Pilo and Frey, 2011; WHO, 1998). In humans, the clinical manifestation is dependent upon the route of infection. Three major forms are described: the cutaneous, the gastro-intestinal and the pulmonary form (WHO, 1998). Lately, injectional anthrax (IA) was observed in patients after injection of drug contaminated with *B. anthracis* spores (Hanczaruk et al., 2014; Ringertz et al., 2000).

B. anthracis belongs to the *B. cereus* sensu *lato* group (Okinaka and Keim, 2016). Species included in this group are distinguished by phenotypic differences, host species predilection, clinical manifestation and ecological niches. However, old molecular typing methods did not allow to clearly distinguish them suggesting to consider them as a "single species" (Helgason et al., 2000). Subsequently, analyses of the gene flow across the *B. cereus* sensu *lato* group revealed that *B. anthracis* is clonal and, discerning from the other species (Didelot et al., 2009). In clinical settings, the identification of *B. anthracis* is based on phenotypic and genotypic characteristics. *B. anthracis* is sensitive to penicillin, phage γ and produces a capsule (WHO, 2008). However, unusual virulent strains that cause anthrax but showing phenotypic traits of *B.*

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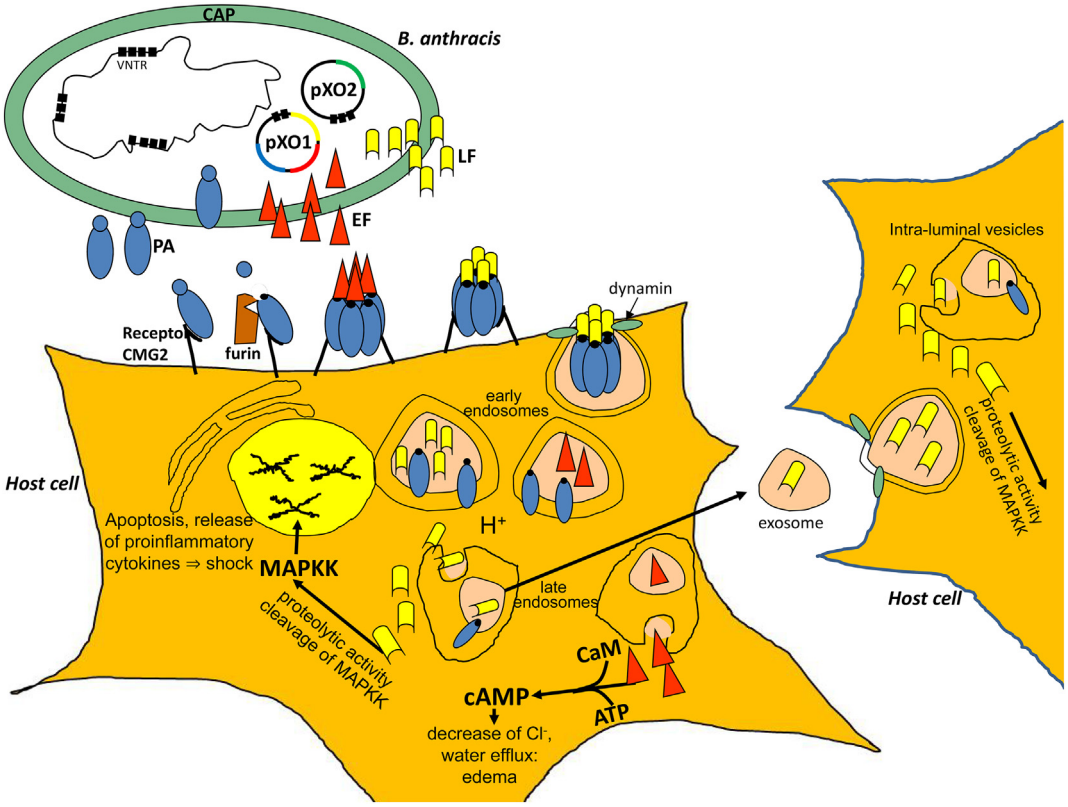

Fig. 1. Overview of the molecular mechanisms of virulence of *Bacillus anthracis*.
B. anthracis (upper left) synthesizes the 4 major virulence factors: the lethal toxin zinc metalloprotease (LF), the edema factor calmodulin-dependent adenylate cyclase (EF) and the adhesive subunit of the two toxins named 'protective antigen' (PA) all of which are encoded on plasmid pXO1, and the poly-D-glutamic acid polymer capsule (CAP) that allows survival of the bacterium in macrophages encoded on plasmid pXO2. PA binds to the receptors on the host cell, CMG2 and TEM8, and is subsequently cleaved by furin, allowing multimerization and binding of LF or EF. The PA:EF and PA:LF complexes are then internalized by endocytosis in a dynamine-clathrin dependent way. In the acid environment of the endosomes, the PA:EF and PA:LF complexes change conformation in order to dissolve the complexes, transit to late endosomes and release the toxic subunits, which exert their activities by overproduction of cAMP by EF and apoptosis, release of pro-inflammatory cytokines subsequent shock by proteolytic cleavage of MAPKK by LF. Part of the toxins remain in intra-luminal vesicles that are able to transfer as exosomes and to be incorporated to non-infected cells, where the toxins can be stored for long term, release and exert their activity long after the infection has taken place. Abbreviations: CAP, capsule; PA, protective antigen; EF, edema factor; LF, lethal factor; VNTR, variable number tandem repeat; MAPKK mitogen-activated protein kinase kinase; CaM, calmodulin; cAMP, cyclic adenosine 3′ 5′ mono phosphate; CMG2, capillary morphogenesis gene 2. Figure adapted from (Pilo and Frey, 2011).

cereus have been isolated. These strains make the classic identification of the etiological agent of anthrax more difficult (Hoffmaster et al., 2006; Hoffmaster et al., 2004; Irenge and Gala, 2012; Klee et al., 2010; Leendertz et al., 2006; Marston et al., 2016; WHO, 2008; Wright et al., 2011). Virulence of *B. anthracis* is determined by two virulence plasmids, pXO1 and pXO2, that can be targeted by specific PCRs. The plasmid pXO1 encodes for two binary toxins: the lethal factor (LF) and the edema factor (EF) and an adhesion subunit termed the protective antigen (PA) that is common to both lethal factor and edema factor. Plasmid pXO2 carries an operon, *capBCDAE,* for the production of a poly-γ-D-glutamic acid capsule (Fig. 1).

B. anthracis has the peculiar biological feature to alternate between a vegetative phase and a spore phase. In the environment, *B. anthracis* occurs principally as spores where it is metabolically dormant and does not replicate over long periods. Once the spores are taken up by the host, they germinate and vegetative cells multiply in the host. Within the host, *B. anthracis* is able to avoid clearing by the immune system thanks to its toxins and capsule. Under these conditions, replication lasts for a relatively short period of 20–40 generations until the death of the host or clearance of vegetative bacteria by therapeutic agents (Keim et al., 2004). Exceptionally, *B. anthracis* can grow in rich soil but tends to lose the virulence plasmid pXO2, indicating that this virulence attribute not only grants the ability to infect mammals but also confers species host restriction (Saile and Koehler, 2006). In contrast to most other bacteria with similar generation times, *B. anthracis* evolves very slowly due to the long dormant periods as spores. Due to this slow evolution and the lack of lateral gene transfer, *B. anthracis* is genetically and phenotypically highly homogeneous. Hence, individual strains or isolates cannot be subtyped by old methods such as serotyping, biochemical reactions or phage typing. However, recently developed rapid sequencing methods have resulted in the availability of full genome sequences of hundreds of *B. anthracis* strains. These data allow the identification of single strains of *B. anthracis* in outbreak investigations and forensic settings and give a constantly improving and detailed picture of the population genetics of *B. anthracis* around the world.

2. Taxonomy

B. anthracis belongs to the phylum *Firmicutes*, the family *Bacillaceae,* the genus *Bacillus* and the *Bacillus cereus* group. The latter consists of six members: *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis, B. weihenstephanensis* and *B. cytotoxicus* (Guinebretiere et al., 2013; Okinaka and Keim, 2016). These species are closely related and have been traditionally identified based on phenotypic characteristics, pathogenicity, clinical symptoms, host preference and ecological niche (Rasko et al., 2005). Most of these species are of economic, environmental, medical and biodefence importance. The chromosomes of the different members of the *B. cereus* group show a high level of

synteny and protein similarity with little difference in gene content (Okinaka and Keim, 2016; Rasko et al., 2005). The main virulence attributes of the species of the *B. cereus* group are host specific toxins and capsular carbohydrates that allow bacteria to avoid clearance by the host's immune defence. They are generally encoded on plasmids, and define host specificity and consequently delineate the bacterial species. The identification of individual species of the *B. cereus* group is therefore complex, and some isolates display unusual biochemical and/or physiological properties that confound their accurate differentiation (Klee et al., 2006; Beesley et al., 2010). Phylogenetically, the *B. cereus* group of bacteria is divided into 3 distinct clades that show partial barriers to inter-clade gene flow. Strains of *B. anthracis* that are highly clonal are restricted to clade 1 together with the highly virulent emetic toxin (cereluid synthethase) producing *B. cereus* strains. Other virulent *B. cereus* strains and the insect pathogen *B. thuringiensis* are found in clade 2. Clade 3 contains environmental, mostly non-pathogenic *B. cereus*, *B. mycoides* and *B. weihenstephanensis*. (Priest et al., 2004; Ko et al., 2004; Helgason et al., 2004; Didelot et al., 2009). The recently described species *B. cytotoxicus* seems to form a new clade as identified by 16S rRNA gene sequence based phylogeny (Guinebretiere et al., 2013; Okinaka and Keim, 2016).

B. anthracis can be identified using a battery of specific bacteriological tests that examine colony morphology, capsule staining, lack of hemolysis, sensitivity to γ-phage, sensitivity to penicillin and motility (WHO, 1998). Nevertheless, some strains may show variability in terms of phenotype, complicating the exact identification and differentiation between *B. anthracis* and other types of *B. cereus*, although these two species show different pathological manifestations (Klee et al., 2006; Beesley et al., 2010). Nonetheless, strains with characteristics of *B. cereus* have been isolated from animals with clinical anthrax (Antonation et al., 2016; Hoffmann et al., 2017; Hoffmaster et al., 2006; Hoffmaster et al., 2004; Klee et al., 2006; Pilo et al., 2011). Hence, several established genetic markers for *B. anthracis*, such as the chromosomal locus Ba813 (Patra et al., 1996) or the gene *sap* encoding the S-layer protein (Ryu et al., 2003) can result in ambiguous diagnostic identification of *B. anthracis* (Ramisse et al., 1999).

The key virulence determinants that differentiate pathogenic species of the *B. cereus* group in their pathogenicity and host specificity represent relatively small portions of their genomes. These genome segments are harbored by mobile elements and were likely acquired by gene transfer from other species rather than evolved by adaptation in their respective hosts. Virulence of *B. anthracis* is determined by two plasmids named pXO1 (toxin plasmid) and pXO2 (capsule plasmid) (Fig. 1). Under certain circumstances, these plasmids can be transferred among related species, particularly among *B. cereus* clade 1 or are found in this clade as similar plasmids (Didelot et al., 2009; Hoffmaster et al., 2004; Hu et al., 2009; Klee et al., 2006; Ruhfel et al., 1984).

3. Mechanisms of pathogenicity

The main virulence factors of the vegetative form of *B. anthracis* are the poly-γ-D-glutamic acid capsule and the tripartite anthrax-toxin. The poly-γ-D-glutamic acid capsule encoded on the virulence plasmid pXO2 is of low immunogenicity and confers resistance against phagocytosis and the complement system, rendering the infectious agent invulnerable to the host's immune system (Makino et al., 2002).

The tripartite toxin is composed of two AB–type toxins. The first one is composed of the adhesion subunit 'protective antigen' PA plus the lethal toxin subunit LF, a metalloprotease. The second consists of PA plus the edema toxin subunit EF, a highly efficient calmodulin-dependent adenylyl cyclase (Fig. 1). In order to exert their toxic biochemical activities at the targets, the toxins need to gain access to the cell cytoplasm. This task is performed by hijacking cellular pathways with the help of two main receptors that are recognized by PA: the capillary morphogenesis genes 2 product (CMG2 or ANTXR2) and the tumor endothelial marker 8 (TEM8 or ANTXR1) (Bradley et al., 2001; Scobie et al., 2003). Both are integrin like proteins that bind to the extracellular matrix proteins such as collagens and fibronectin and have a van Willebrand factor A domain, an Ig-like domain plus a helical trans membrane domain (Deuquet et al., 2012). Knockout-mice deleted for the CMG2 gene are fully resistant to anthrax toxin challenge indicating that CMG2 is the main receptor for the anthrax toxins (Liu et al., 2012). Once PA is bound to the receptors at the host cell surface, the 83 kDa PA is cleaved by a host furin-like protease to produce a shorter 63 kDa form that assembles into heptamers or octamers in membrane lipid rafts to which LF and EF can bind (Abrami et al., 2003) (Fig. 1). The complexes PA:LF and PA:EF are internalized by dynamin-clathrin-mediated endocytosis (Abrami et al., 2010). After internalization from the cell surface PA undergoes a conformational change triggered by the acidic (H^+) environment in endosomes forming pores to translocate the enzymatic subunits EF and LF to the cytoplasm where they exert their toxic activity, or to an intermediary state in intraluminal vesicles. In the cytoplasm LF, the zinc dependent metalloprotease, inactivates members of the MAPKK kinase family (MEK) by proteolytic cleavage which leads to excessive release of cytokines, apoptosis, and finally to necrosis and hypoxia (Duesbery et al., 1998).

The edema factor EF, which reaches the same compartments as LF using PA (Fig. 1), is a highly efficient calmodulin-dependent adenylyl cyclase that is about 1000 times more active than mammalian adenylyl cyclase to convert ATP to c-AMP. At high concentrations, c-AMP results in the decrease of chloride ions and a strongly increased water efflux from the affected cells (Drum et al., 2000). This leads to massive edema that is typical in cutaneous anthrax.

In the initial phases of the infection, both LF and EF target and disable myeloid cells, such as macrophages and neutrophils, blocking the host's immune system and allowing the infection to progress to an acute state with fever, sore throat, diarrhea and vomiting. The later stages of anthrax are favored by the fact that the toxins that have reached intraluminal vesicles remain protected from lysosomal enzymes, hence allowing long-term storage of the potent active toxins (Abrami et al., 2013) (Fig. 1). This long-term storage of active enzymes in intraluminal vesicles and their long lasting release that is possible even in absence of bacteria are important characteristic of the high virulence of *B. anthracis*. It seems to be the reason why patients even after successful elimination of bacteria by treatment with antibiotics still succumb to the disease dying from a systemic infection when treatment is administrated too late (Abrami et al., 2013).

4. Molecular typing methods

Bacterial genomes are dynamic. Numerous molecular mechanisms shape the evolution of bacteria and various genetic processes generate genomic diversity. They are mainly based on acquisition or loss of genes, genome rearrangements and accumulation of mutations appearing during DNA replication resulting in the genomic heterogeneity of bacterial populations (Davis and Isberg, 2016; Fraser-Liggett, 2005). At the level of strains, genomic variations can lead to specific phenotypic traits. Initially, typing of bacterial strains was tested phenotypically but molecular technics were rapidly included in the panel of possibilities to distinguish strains (Li et al., 2009). In the last decades, improvements achieved in DNA sequencing technologies and the development of bioinformatics tools to analyze data revolutionized investigations of intra-species diversity, even for genetically homogeneous species like *Yersinia pestis*, *Salmonella enterica* Typhi, *Mycobacterium leprae* and *Bacillus anthracis* (Benjak et al., 2018; Bochkareva et al., 2018; Fournier et al., 2014; Holt et al., 2008; Schuenemann et al., 2018; Van Ert et al., 2007). Nowadays, genomic characterization of strains is applied beyond clinical bacteriology and epidemiology in fields such as vaccine design (Cafardi et al., 2013), food industry (Douillard and de Vos, 2014) and forensic science (Gonzalez et al., 2017).

Early attempts to type *B. anthracis* strains were hampered because

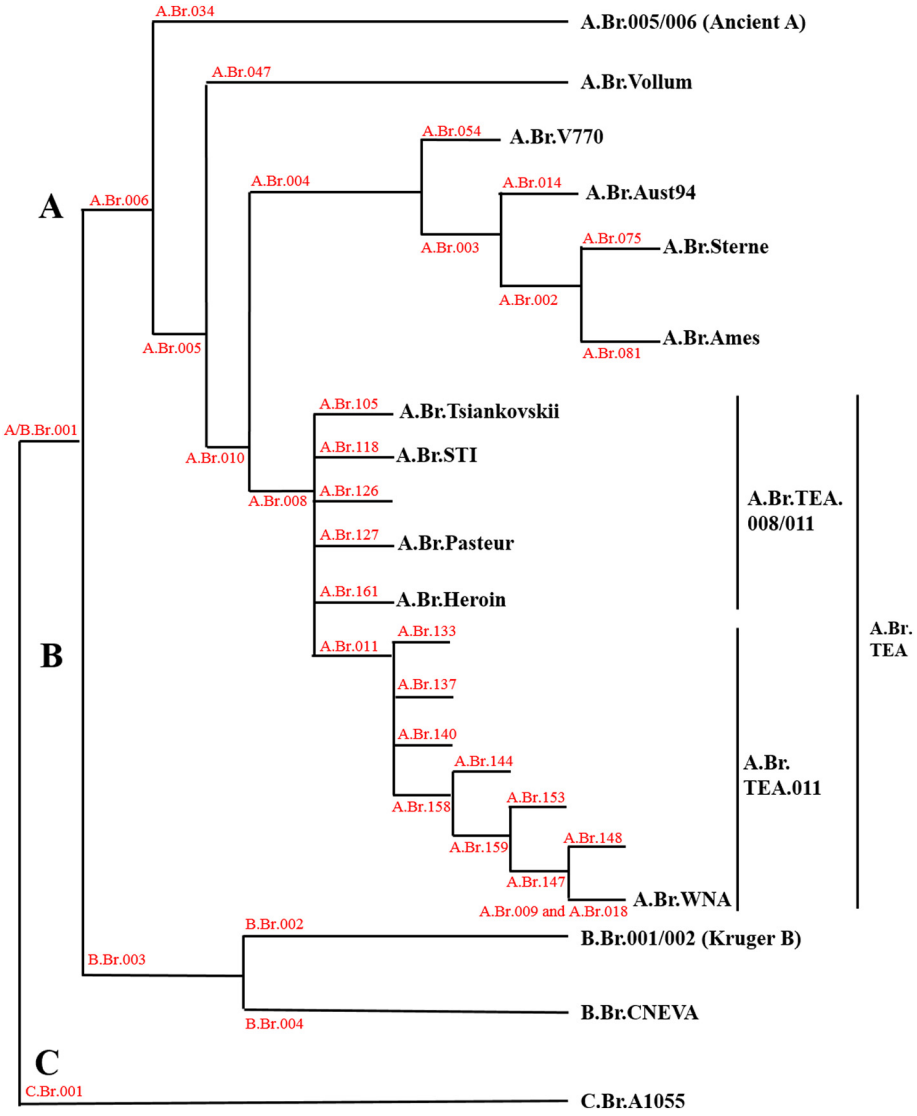

Fig. 2. Schematic tree summarizing the nomenclature based on canSNPs of *B. anthracis*. The three major lineages of *B. anthracis*, A, B and C, are shown in bold capital letters. The canSNPs are shown in red. Branch lengths are not scaled. Data adapted from (Sahl et al., 2016). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this bacterium is genetically very homogeneous and underwent clonal evolution compared to the other species belonging to the *B. cereus* sensu *lato* group (Ehling-Schulz et al., 2005; Keim et al., 2000). Common genetic tools to differentiate bacterial strains were not adequate for this species (Harrell et al., 1995) before the advent of high-throughput DNA sequencing methods. Typing schemes based on Variable Number Tandem Repeats (VNTR) provided the first step in distinguishing individual *B. anthracis* strains, initially using the repeats in the gene named variable region with repetitive sequence *vrrA* (Andersen et al., 1996; Henderson et al., 1995). Additional tandem repeats were subsequently identified leading to the development of a protocol to perform multiple-locus VNTR analysis (MLVA) (Keim et al., 1997; Keim et al., 1999; Keim et al., 2000). The assay was initially developed for eight markers (MLVA8): six chromosomal (*vrrA*, *vrrB1*, *vrrB2*, *vrrC1*, *vrrC2* and CG3) and two plasmidic markers (pXO1-aat and pXO2-at). Successively, supplementary markers were identified and the MLVA scheme was extended to MLVA15 (MLVA8 markers plus Bavntr12, Bavntr16, Bavntr17, Bavntr19, Bavntr23, Bavntr32, Bavntr35) (Van Ert et al., 2007), MLVA20 (Le Fleche et al., 2001) and MLVA25 (Lista et al., 2006). All described VNTR markers were lately merged resulting in MLVA31 (Beyer et al., 2012; Braun et al., 2015; Grunow et al., 2013; Rume et al., 2016; Thierry et al., 2014). An additional protocol using 22 single nucleotide repeat (SNR) markers to achieve higher resolution that might be required for epidemiological studies was described (Kenefic et al., 2008a; Stratilo and Bader, 2012; Stratilo et al., 2006). MLVA allows high resolution for typing strains (Keim et al., 2004). However, this method is prone to homoplasy and thus interferes with phylogenetic studies (Keim et al., 2004). Reverse mutations causing homoplasy are more likely to occur in VNTR loci with lower diversity, due to the structure of the repeats that cause a higher error rate by polymerases and to the fact that many VNTRs are not in coding or regulatory sequences and therefore are not subject to selective pressure (Reyes et al., 2012). Furthermore, it must be taken into account that VNTR analysis techniques can cause homoplasy problems in PCR-based assays that strongly affect the inference of recent population history (Wan et al., 2004). VNTR-based methods are hence limited for applications that strongly rely on evolutionary interference such as epidemiological and forensic studies.

High-throughput DNA sequencing methods improved whole genome sequencing (WGS) and the detection of single nucleotide polymorphisms (SNPs) (Pearson et al., 2004). WGS of a large number of *B. anthracis* strains showed that SNPs are evolutionary stable. Thus

representative non-homoplastic SNPs, named canonical SNPs (canSNPs), were selected allowing branch points and clades to be accurately defined for phylogenetic studies. A worldwide collection of 1033 strains of *B. anthracis* was tested with a set of 13 canSNP markers. The strains could be allocated to three major lineages: A, B and C. Within these lineages, 12 sublineages / subgroups (lineage A: A.Br.001/002, A.Br.Ames, A.Br.Aust94, A.Br.003/004, A.Br.Vollum, A.Br.005/006 [=Ancient A], A.Br.008/009 [=Transeurasian: TEA], A.Br.WNA [=Western North American], B: B.Br.Kruger, B.Br.001/002, B.Br.CNEVA and lineage C: C.Br.A1055) were identified (Van Ert et al., 2007). A supplementary canSNP was later identified dividing the group A.Br.008/009 in A.Br.008/011 and A.Br.011/009 (Marston et al., 2011). These studies also delineated the first comprehensive and significant phylogeographical distribution of *B. anthracis* strains.

The number of samples and their diversity are crucial to achieve the most precise representation of phylogenetic trees and geographical distribution. Numerous national collections of *B. anthracis* strains were tested by canSNP typing and/or MLVA (Aikembayev et al., 2010; Antwerpen et al., 2011; Derzelle et al., 2011; Mao et al., 2016; Pilo et al., 2008; Simonson et al., 2009). Selected strains from those collections were fully sequenced and led to the identification of additional canSNPs (Derzelle et al., 2016a; Rume et al., 2016) and to better understand the evolution and distribution of *B. anthracis* strains, such as the A.Br.Ames lineage (Kenefic et al., 2008b) and the A.Br.WNA lineage (Kenefic et al., 2009). The discovery of new canSNPs leads to a finer resolution of phylogenetic trees by adding new branches resulting in the splitting of existing clades into subclades of existing clades.

In order to circumvent phylogenetic discovery bias, a nested hierarchical fingerprinting called progressive hierarchical resolving assays using nucleic acids (PHRANA), based on the progressive analysis of VNTRs including SNRs (Kenefic et al., 2008a; Stratilo et al., 2006) and canSNPs (Keim et al., 2004; Van Ert et al., 2007) was developed. PHRANA allowed higher typing resolution of strains. However, PHRANA is now mostly replaced by whole genome SNP analysis that provides full and high resolution among isolates.

5. Phylogeography, population structure and dissemination of *B. anthracis*

The phylogenetic structure of *B. anthracis* was recently defined more precisely (Sahl et al., 2016). For clarity purposes, new and old designations of clusters and subclusters are mentioned. In the current state of knowledge, *B. anthracis* is divided in 3 major clades: A, B and C (Fig. 2). Clade A is broadly dispersed throughout the world, while clades B and C seem to display a narrower geographical distribution (Sahl et al., 2016; Van Ert et al., 2007; Vergnaud et al., 2016). Although clade A is the most dispersed branch across the world, subgroups show particular geographical patterns. The clade A is subdivided in four monophyletic subclades: A.Br.005/006 (Ancient A, A.Br.034), A.Br.Vollum (A.Br.005/010, A.Br.047), A.Br.010/008 (A.Br.004) and A.Br.010/004 (A.Br.TEA, A.Br.008) (Fig. 2). Group A.Br.005/006 is basal to the other subclades and contains strains isolated in Africa (Girault et al., 2014; Hang'Ombe et al., 2012; Ohnishi et al., 2014; Sahl et al., 2016). A.Br. Vollum (A.Br.005/010 or A.Br. 047) is most widely dispersed geographically. A.Br.Vollum seems to be dominant in Pakistan and Afghanistan but it is also found in Western China (Derzelle et al., 2016a; Price et al., 2012). A.Br.Vollum strains were frequently isolated (and are still occasionally) from wool factories in Europe and Northern America and also other parts of the world, assumingly imported via contaminated raw wool from the area Northern Pakistan – Afghanistan (Aikembayev et al., 2010; Derzelle et al., 2015; Khmaladze et al., 2014; Pilo et al., 2008; Van Ert et al., 2007; Wattiau et al., 2008).

Subclade A.Br.008 (A.Br.TEA) is highly prevalent in China and widely spread across Asia and Europe. In Europe, A.Br.TEA is present together with the 'European' cluster B.Br.004. (B.Br.CNEVA) and subgroup A.Br.Sterne. Sublineage A.Br.014 (A.Br.Aust94) is spread in Western China, Turkey and India, while A.Br.081 (A.Br.Ames) is more prevalent in Eastern and Central China. A.Br.034 (A.Br.005/006, Ancient A) is largely restricted to Southern Africa. Strains isolated in the American continent belong mainly to A.Br. 009 (A.Br.WNA) in North America and to A.Br.054 (A.Br.V770) in South America (Fig. 2). In comparison, lineages B and C show limited distribution. C is only detected in North America and B in Europe and South Africa (Van Ert et al., 2007).

Cluster A.Br.010/008 is subdivided in three subclades: A.Br.054 (V770), A.Br.003/014 (Sterne/Ames subclade; further subdivided in A.Br.075 [Sterne] and A.Br.081 [Ames]) and A.Br. 003/002 (Aust94) (Fig. 2) (Sahl et al., 2016). A.Br.054 is broadly spread as it was found in the American continent, Africa and Europe (Sahl et al., 2016). This subclade includes attenuated strains produced in the USA in the '60s (Wright et al., 1962). A.Br.003/014 is thought to have origins in China and subsequently spread across the world (Simonson et al., 2009). This subclade is found in Asia, the American continent and in Europe (Sahl et al., 2016; Van Ert et al., 2007). A.Br.003/002 is present on all continents (Khmaladze et al., 2014; Van Ert et al., 2007). Subclade A.Br.TEA of the branch A is further divided in A.Br.008/011 (A.Br.TEA Br.008/011), A.Br.WNA. and A.Br.011/009 (TEA Br.011) (Figs. 2 and 3) (Marston et al., 2011; Sahl et al., 2016). A.Br.TEA is highly branching with several direct descendant subclades (Fig. 2) and with generally only few SNPs developing between branches (Sahl et al., 2016; Vergnaud et al., 2016).

A.Br.WNA. is found largely in North America (Figs. 2 and 3). It was used to argue for an ungulate transmission model with an estimated infection/death/year rate of 0.28 generations per year, based on a study of 13 consecutive anthrax outbreaks in cattle in northern Canada between 1962 and 1991 (Dragon and Elkin, 2001; Kenefic et al., 2009). Analysis of a large number of *B. anthracis* strains with precise geographical data of isolation led to estimate the dispersion of clade A reaching the Northern American continent in the late Pleistocene epoch, about 13'000 years ago via the former Bering Land Bridge (that was cleared later on by receding glaciers) and progressing as subclade A.Br.WNA from the Northwest Territories and Alberta, Canada, southwards to the southern states of USA and to California (Kenefic et al., 2009). Subsequently, the inclusion of whole genome sequences of European and African A.Br.TEA strains showed a common origin of A.Br.WNA and French A.Br.TEA strains that caused the authors to hypothesize about a much more recent split about 500 years ago (Girault, 2015; Vergnaud et al., 2016) (Fig. 3). Based on these assumptions the authors proposed an alternative model of dissemination of A.Br.WNA to North America, due to Breton explorers and emigrants from Northern France (Jacques Cartier 1541, Samuel de Champlain 1608) that reached the Gulf of St. Lawrence and settled along the St. Lawrence River founding the cities of Quebec and Montréal (Girault, 2015; Vergnaud et al., 2016). Although it is known that explorers and settlers brought along their animals with their chattels and might have disseminated anthrax by contaminated animals, there is currently no evidence of anthrax being ecologically established in this area, which would argument against this hypothesis. Furthermore, an introduction of subclade A.Br.WNA into South-Eastern Canada is not consistent with the phylogenetic pattern of *B. anthracis* strains from Northern America as the most basal A.Br.WNA subclade strains were isolated in the North-West of the continent (Kenefic et al., 2009; Vergnaud et al., 2016). The nearest relationship of strains isolated from Western Africa to French strains of TEA (but also to Italian strains (Sahl et al., 2016) (Fig. 3) was taken as argument that migrants of North France moving to Western Africa, at about the same time as migration to South-Eastern Canada, might have disseminated anthrax to Senegal and today's The Gambia (Girault, 2015; Vergnaud et al., 2016). The disagreement in timing between the models of Kenefic and collaborators (Kenefic et al., 2009; Vergnaud et al., 2016) and that of Girault and Vergnaud (Girault, 2015; Vergnaud et al., 2016) seems be due to the fact that the latter used relatively short branches of the phylogeny of French *B. anthracis* TEA

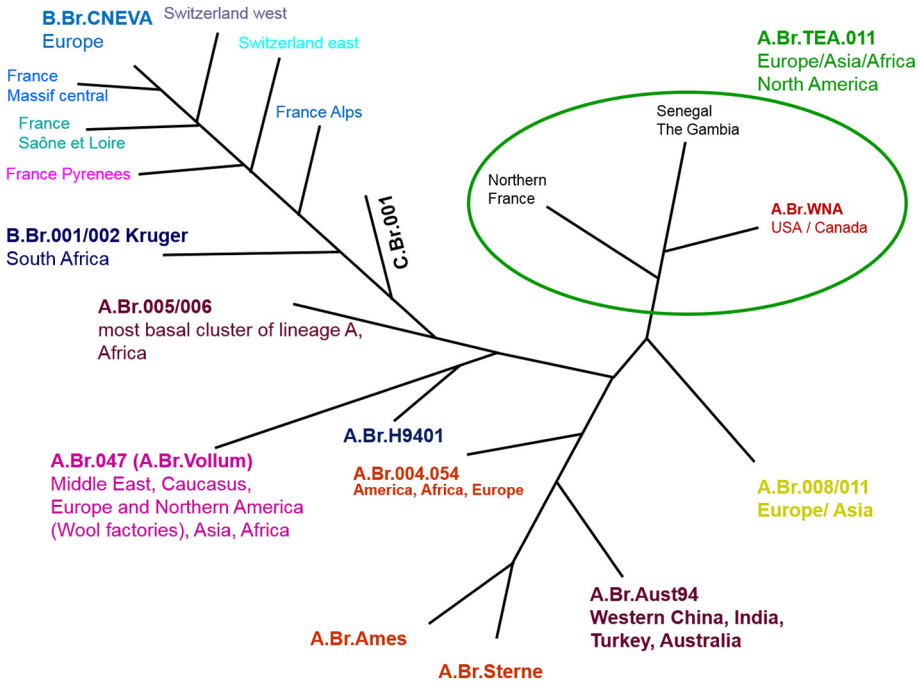

Fig. 3. Schematic tree summarizing the latest data on phylogeography of worldwide *B. anthracis* strains.
The different canSNP groups are shown in different colors. The three major clades A, B and C are indicated by the first capital letter of the designation of the major canSNP groups. The large clade A is represented on the right and lower part of the figure. Clade B is on the upper left part and the small clade C is in black in the middle. Branch lengths are not scaled. The geographic locations of the French and Swiss sub-groups of clade B are specifically given to illustrate the narrow geographic clustering. Data are compiled from (Derzelle et al., 2016a, 2016b; Derzelle et al., 2011; Girault, 2015; Girault et al., 2014; Vergnaud et al., 2016).

strains as a basis for their timing. This assumes that longer branches, as were described for A.Br.WNA. strains (Kenefic et al., 2009; Vergnaud et al., 2016), would represent accelerated evolution. However, recent work on evolutionary timing indicates that branch lengths are not adequate for the determination of timing of divergence of the different clades and subclades (Sahl et al., 2016). In particular, short branches and polytomies at critical positions in the phylogenic structure are prone to blur the determination of evolutionary timing (Sahl et al., 2016).

Clade B represents around 10% of the strains analyzed worldwide (Sahl et al., 2016). This lineage shows a limited geographical distribution and is divided in two subclades: B.Br.CNEVA (B.Br.004) and B.Br.001/002 (B.Br.002) (Derzelle et al., 2016b; Sahl et al., 2016; Van Ert et al., 2007). B.Br.CNEVA is found in Europe only and coexists with strains belonging to the clade A resulting in two overlapping clades in Europe (Derzelle et al., 2011; Garofolo et al., 2011; Gierczynski et al., 2009; Pilo et al., 2008; Van Ert et al., 2007). Clades A and B initially showed a long common history before the first bifurcation is observed within each clade separating B.Br.001/002 from B.Br.CNEVA and separation of clade A.Br.005/006 from the rest of clade A (Fig. 3). These divergences were estimated to have occured about the time of the emergence of agriculture and farming in the Fertile Crescent region and subsequent human migrations early farmers into Europa over the following millenniums (Derzelle et al., 2016b). In contrast to clade A, which spread around the world facilitated strongly by international trade of animal hair and skin products, strains from B.Br.CNEVA have only been reported from France, Southern Germany, Switzerland, Northern Italy, Bosnia and Herzegovina, Croatia, Slovenia, Slovakia and Poland (Girault, 2015; Vergnaud et al., 2016). This represents a transalpine axis constituted by pastoral valleys with rich grassland where cattle breeding might have provided a favorable environment for spore survival and host-propagation of *B. anthracis*. As depicted in Fig. 3, subclustering of strains of B.Br.CNEVA are concordant with geographical patterns (Derzelle et al., 2016b; Derzelle et al., 2011; Girault et al., 2014). These latter strongly correspond to specific cattle breeds that were kept isolated in these locations over many centuries (Derzelle et al., 2016b; Derzelle et al., 2011; Girault et al., 2014). Main breeds of specific areas are: in Saône et Loire: Charolaise, in the French Massif Central: Limousine, in French Alps: Tarantaise and Abondance, in French Pyrenees: Cascogne and Lourdais, in Western Switzerland: Simmental and in Eastern Switzerland Swiss Brown. All these cattle breeds were kept traditionally and considered as local heritage for centuries and were not exchanged. This geographic isolation might have contributed to the genetic diversity observed among B.Br.CNEVA (Derzelle et al., 2016b). This is in strong contrast to strains of clade A, which in Europe do not correlate to areas with specific cattle breeds. Hence, strains of clade B might be older in Europe than clade A strains assumingly spread later on by international trade. Subcluster B.Br.001/002 is ecologically established in South Africa and might have been brought by early settlers (Smith et al., 2000).

Clade C seems to occur extremely infrequently since only three strains from North America were isolated and analyzed up to present (Sahl et al., 2016; Van Ert et al., 2007).

6. Forensic applications

Beyond the information gained at the level of geographical distribution of strains and evolution of *B. anthracis*, the molecular tools developed to analyze intraspecies diversity proved valuable for forensic investigations, even retrospectively. In these cases, molecular analyses provided essential information to solve unanswered investigative questions. 1) the anthrax sugar lumps from 1917 (Antwerpen et al., 2017), 2) the Sverdlovsk outbreak in 1979 (Sahl et al., 2016), 3) the Kameido incident in 1993 (Takahashi et al., 2004), 4) the Amerithrax attack (Rasko et al., 2011) and 5) the outbreaks of injectional anthrax (IA) (Keim et al., 2015).

The first example dates from the First World War. A German spy, the Baron Otto von Rosen, was arrested in Norway in 1917 for suspicion of espionage and sabotage in possession of sugar lumps containing *B. anthracis* (Redmond et al., 1998). The lumps were deposited afterwards at the police museum in Trondheim, Norway. In 1997, they were sent to the Defence Evaluation Research Agency, Chemical and Biological Defence, in Porton Down, UK, for analysis (Antwerpen et al., 2017; Redmond et al., 1998). After sub-culturing and enrichment efforts, four colonies, phenotypically consistent with *B. anthracis,* were recovered (Redmond et al., 1998). Subsequent genomic analyses showed that the colonies isolated from the samples belong to the subcluster A.Br.Ames and that they have the same MLVA profile than the strain Ames

Ancestor. Additionally, in silico analysis of genomes revealed only two nonsynonymous SNPs with strain Ames Ancestor (Antwerpen et al., 2017). Since this strain was isolated from a cow in the USA in 1981 and it is a common laboratory strain, the colonies isolated from the sample of the Trondheim police museum were considered to be laboratory contaminations (Ravel et al., 2009).

The following example resulted in one of the larger outbreaks of inhalational anthrax, affecting humans and animals, that occurred in 1979 in Sverdlovsk, former USSR (today Jekaterinburg) (Abramova et al., 1993; Meselson et al., 1994). This epidemic raised the suspicion of an accidental release of *B. anthracis* spores from a military site. In former USSR, the event was first reported as an outbreak of gastrointestinal anthrax caused by naturally contaminated meat form diseased animals that were slaughtered in the adjacent rural area (Meselson et al., 1994). However, pathological evidence suggested cases of inhalational anthrax (Abramova et al., 1993). The presence of *B. anthracis* was later confirmed by PCR in formalin-fixed paraffin embedded (FFPE) tissues of victims (Jackson et al., 1998). CanSNP analysis assigned the sample to clade A.Br.008/009 (Okinaka et al., 2008). Direct sequencing of FFPE samples originating from two patients refined the phylogenetic position of the infecting strain: A.Br.008/009 more precisely the TEA subclade A.Br.008/011 (Sahl et al., 2016). The results position the Sverdlovsk strain in a clade comprising wild type Eurasian strain, including two Asian live vaccine strains. The authors of this study also examined the possibility of genetic manipulations but did not find evidence for this strain. Additionally, the similarity of the Sverdlovsk strain to genomic sequences with wild type strains suggest limited sub-culturing of the strain used (Sahl et al., 2016).

The third example is a case that occurred in Japan in late June 1993 (Sugishima, 2003). The Aum Shinrikyo tried to release spores of *B. anthracis* from the roof of their headquarter in Tokyo. The event was noticed because of “foul odors” in the neighborhood (Takahashi et al., 2004). Despite dozens of complaints reporting loss of appetite, nausea, and vomiting from residents, none of this cases developed human anthrax. A sample, collected at the site, was subsequently analyzed. After isolation of *B. anthracis* colonies, the isolates were subjected to MLVA. All isolates showed the same VNTRs profile as strain Sterne (Sahl et al., 2016; Sterne, 1939). This strain is widely used as a life vaccine in veterinary medicine (Sterne, 1988). Both, strain Sterne and the isolates from the intentional release in Tokyo lack plasmid pXO2 encoding for the capsule. The fact that no anthrax cases were reported in this attack is assumed to be related to the pXO2-deficient life vaccine strain that was released and that is known to be safe for cattle and hence seems not to be virulent or of only low virulence to humans.

Following September 11, 2001, the US suffered attacks with spores of *B. anthracis* sent in letters to two senators and several news agencies via mail. At least 22 victims developed anthrax (11 cutaneous and 11 inhalational), among them, five died of pulmonary anthrax (Jernigan et al., 2002; mailings, 2011). Using VNTR and SNP analysis the isolates could be categorized as strain Ames (Hoffmaster et al., 2002). However, the investigating scientists noticed colonies displaying minor morphological variations present from samples of the different letters. The same variants were present in all the letters. After full genome sequencing, they could attribute genomic differences to the different morphological types observed (Rasko et al., 2011). These genomic signatures were unique enough to trace back the source of the spores to a specific laboratory batch. These results confirmed that the strain used for the attacks was a laboratory strain and not a strain circulating in natural settings.

The last example concerns a new form of anthrax that was recently described. The first death due to *B. anthracis* in an injectional heroin drug user was reported in 2000 (Ringertz et al., 2000). Two outbreaks of IA followed in 2009–2010 (UK, Germany) and in 2012–2013 (Denmark, France, Germany, UK), respectively (Berger et al., 2014). This manifestation of the disease was of major concern because of the poor clinical outcome (Booth et al., 2010; Hanczaruk et al., 2014; Powell et al., 2011). Forensic investigations to determine the source of the heroin contamination were extremely complicated because of the illegality related to heroin production, distribution and use (Team, 2011). Attempts to isolate strains from drug batches were unsuccessful but some isolates could be retrieved from patients and submitted to molecular analysis (Hanczaruk et al., 2014; Team, 2011). These strains were shown to belong to the TEA lineage clustering with strains from Turkey (Hanczaruk et al., 2014; Price et al., 2012). In a subsequent study, Keim and colleagues fully sequenced 58 strains from 36 patients to enhance the resolution of the typing (Keim et al., 2015). By screening of 1293 isolates they could show that heroin contaminations occurred during at least two events possibly during transportation via the Middle East to Europe.

7. *B. cereus* non *anthracis* strains causing anthrax

In early 2000 an anthrax-like disease, in humans and animals, caused by non-*B. anthracis* strains was described (Antonation et al., 2016; Hoffmaster et al., 2006; Hoffmaster et al., 2004; Klee et al., 2010; Klee et al., 2006; Leendertz et al., 2004; Leendertz et al., 2006; Marston et al., 2016; Pilo et al., 2011; Wright et al., 2011). The strains exhibited a combination of phenotypic traits of *B. anthracis* and *B. cereus* and were named *B. cereus* biovar *anthracis* (Table 1) (Antonation et al., 2016; Hoffmaster et al., 2006; Hoffmaster et al., 2004; Klee et al., 2006; Pilo et al., 2011). Interestingly, the strains from the USA were isolated from humans, while the strains isolated in Africa were from animals (Antonation et al., 2016; Hoffmaster et al., 2006; Hoffmaster et al., 2004; Klee et al., 2010; Klee et al., 2006; Leendertz et al., 2004; Leendertz et al., 2006; Marston et al., 2016; Pilo et al., 2011; Wright et al., 2011). *B. cereus* biovar *anthracis* from Africa were shown to

Table 1
Phenotypic traits of *B. cereus* non *anthracis* strains causing anthrax.

Phenotype	Strain										
	G9241	03BB87	03BB102	BcFL2013	03BB108	Elc2	CA	CI	RCA A-363/2	DRC 14-0024-1	JF3964
Hemolysis	+	+	+	+	+	+	−	−	−	−	−
Motility	+	+	+	ND	+	+	+	+	+	−	ND
Gamma phage	R	R	R	R	R	ND	R	R	R	R	R
Capsule	+	+	+	+	−	+	+	+	+[a]	+[a]	+[a]
Penicillin	S	ND	ND	ND	ND	R[b]	R	S	R	R	R
Published in	Hoffmaster et al., 2004	Hoffmaster et al., 2006	Hoffmaster et al., 2006	Marston et al., 2016	Hoffmaster et al., 2006	Wright et al., 2011	Klee et al., 2006	Klee et al., 2006	Antonation et al., 2016	Antonation et al., 2016	Pilo et al., 2011

R: resistant; S: sensitive; ND: not determined.
[a] Suspected because of mucoid appearance of colonies.
[b] Tested only for ampicillin and ceftriaxone resistance.

harbor plasmids similar to pXO1 and pXO2 that are positive by PCR for the respective virulence gene markers of these plasmids (Antonation et al., 2016; Klee et al., 2010; Pilo et al., 2011). In contrast, American strains seem to have a pXO1-like plasmid but not a pXO2-like plasmid (Hoffmaster et al., 2006; Hoffmaster et al., 2004; Marston et al., 2016; Wright et al., 2011). Instead, they possess a second plasmid pBC218, which carries the *bpsX-H* operon encoding for another polysaccharide capsule but does not harbor the *capBCDAE* genes that encode for the poly-γ-D-glutamic acid capsule (Hoffmaster et al., 2004); (Hoffmaster et al., 2006; Oh et al., 2011). This is confirmed phenotypically as American strains do not react with antibodies directed against the poly-γ-D-glutamic acid capsule of *B. anthracis* (Sue et al., 2006). The pXO1-like plasmid pBCXO1 contains the genes *hasABC* encoding for the biosynthesis of a hyaluronic acid capsule and the toxin genes *pagA1, lef1, cya1* (Oh et al., 2011). An inactive *hasACB* operon is also present in the pXO1 plasmid of *B. anthracis*. It is not expressed, due to the presence of a single mutation (Oh et al., 2011; Okinaka et al., 1999). *B. cereus* strain G9241 contains plasmid pBCXO1 with the toxin genes, as well as *hasACB*, providing for hyaluronic acid capsule formation plus plasmid pBC218 harboring the operon *bpsX-H*, specifying the *B. cereus* exopolysaccharide, which produce a second capsule. During infection, *B. cereus* G9241 both *hasACB* and *bpsX-H* capsules are essential for the establishment of anthrax-like disease and resistance to phagocytosis. In contrast, African *B. cereus* biovar *anthracis* strains produce a poly-γ-D-glutamic acid capsule since they carry a pXO2-like plasmid (Antonation et al., 2016; Klee et al., 2006). They also synthetize the hyaluronic acid capsule (Brezillon et al., 2015). Remarkably, curing of the pXO2-like plasmid in these strains did not significantly alter their virulence in mice and guinea pigs (Brezillon et al., 2015). This is a contrasting finding compared to *B. anthracis* in which the loss of pXO2 results in substantial attenuation (Harvill et al., 2005). These findings confirm that the presence of a functional *hasACB* operon providing the hyaluronic acid capsule enables these strains of *B. cereus* biovar *anthracis* to have full virulence in mammals (Brezillon et al., 2015; Oh et al., 2011). While *B. anthracis* is largely limited to arid ecosystems where outbreaks occur mostly in bovine species, anthrax-causing *Bacillus cereus* biovar *anthracis* in Africa is widely present and persistent in tropical rainforest where it is responsible for causing death to a wide range of mammalian hosts and constitutes a particular threat to local chimpanzee populations (Hoffmann et al., 2017).

8. Conclusion

New biochemical and molecular biological data on the binary anthrax toxins reveal their high potency and high specificity to receptors of the capillary morphogenesis genes 2 product (CMG2) and the tumor endothelial marker 8 (TEM8) by means of the adhesive and translocation subunit, the protective antigen PA. Immune cells are the most heavily targeted cells by the lethal toxin LT. By subversion of the cellular compartments, the lethal toxin is able to persist for long periods in intra-luminal vesicles and thus insuring the pathogen an ideal environment to spread its effects to disabled cells. This explains the particular virulence of the anthrax toxins and the unconditional administration of rapid therapy of infected individuals. Based on full genome sequencing of many strains of *B. anthracis* and analysis of single nucleotide polymorphisms, the dissemination of anthrax could be traced back to early origins and shows the important role of domestication of animals, human migration and international trade of animal products as the cause of the worldwide spread of anthrax. While whole genome sequencing results in detailed and robust clade structures of *B. anthracis*, temporal predictions of specific events remain critical in spite of large data sets from high-throughput sequencing. A still rarely reported type of anthrax caused by strains of *B. cereus* biovar *anthracis* in Africa has its proper epidemiology in inhabited areas of tropical rainforests, where it threatens primate populations. The new whole genome sequencing techniques and potent bioinformatics represent powerful means to analyze large numbers of strains in order to unravel epidemics and to implement measures to protect humans, livestock and wildlife populations.

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